

Record: 428382

<u>Accession Number</u>	428382
<u>Borrower</u>	Anne L. Holleran
<u>Organization</u>	1642
<u>Phone</u>	308-8892
<u>Request Date</u>	01/21/2003
<u>JRDAT</u>	2651
<u>Document Type</u>	Journal article
<u>Journal Name</u>	AMERICAN JOURNAL OF PHYSIOLOGY
<u>Journal Location</u>	AGL; LC; CAS; NLM; NSD; NST; PTO
<u>Author</u>	YOREK
<u>volume</u>	274
<u>Issue</u>	1 PT.1
<u>Pages</u>	C58-C71
<u>Year</u>	1998
<u>Publisher</u>	BETHESDA, MD. : ?
<u>Alternate Source</u>	LC  D 01/21/2003
<u>Workdays</u>	-2651
<u>ISSN</u>	0002-9513

# Effect of TNF- $\alpha$ on SMIT mRNA levels and *myo*-inositol accumulation in cultured endothelial cells

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<sup>1</sup>Department of Internal Medicine, Diabetes-Endocrinology Research Center and Veterans Affairs Medical Center, University of Iowa, Iowa City, Iowa 52246; <sup>2</sup>Department of Anatomy and Cell Biology, University of North Texas Health Science Center at Fort Worth and North Texas Eye Research Institute, Fort Worth, Texas 76107; and <sup>3</sup>Veterans Affairs Chicago Healthcare System (Lakeside Division) and Department of Medicine, Northwestern University Medical School, Chicago, Illinois 60611

**Yorek, Mark A., Joyce A. Dunlap, Michael J. Thomas, Patrick R. Cammarata, Cheng Zhou, and William L. Lowe, Jr.** Effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation in cultured endothelial cells. *Am. J. Physiol.* 274 (Cell Physiol. 43): C58–C71, 1998.—Previously we have shown that hyperosmolarity increases Na<sup>+</sup>-*myo*-inositol cotransporter (SMIT) activity and mRNA levels in cultured endothelial cells. Because hyperosmolarity and cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), activate similar signal transduction pathways, we examined the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation. In contrast to the effect of hyperosmolarity, TNF- $\alpha$  caused a time- and concentration-dependent decrease in SMIT mRNA levels and *myo*-inositol accumulation. The effect of TNF- $\alpha$  on *myo*-inositol accumulation was found in large-vessel endothelial cells (derived from the aorta and pulmonary artery) and cerebral microvessel endothelial cells. In bovine aorta and bovine pulmonary artery endothelial cells, TNF- $\alpha$  activated nuclear factor (NF)- $\kappa$ B. TNF- $\alpha$  also increased ceramide levels, and C<sub>2</sub>-ceramide mimicked the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation in bovine aorta endothelial cells. Pyrrolidinedithiocarbamate, genistein, and 7-amino-1-chloro-3-tosylamido-2-hepatanone, compounds that can inhibit NF- $\kappa$ B activation, partially prevented the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. The effect of TNF- $\alpha$  on *myo*-inositol accumulation was also partially prevented by the protein kinase C inhibitor calphostin C but not by staurosporine. These studies demonstrate that TNF- $\alpha$  causes a decrease in SMIT mRNA levels and *myo*-inositol accumulation in cultured endothelial cells, which may be related to the activation of NF- $\kappa$ B.

tumor necrosis factor- $\alpha$ ; sodium *myo*-inositol cotransporter; nuclear factor- $\kappa$ B

TUMOR NECROSIS FACTOR- $\alpha$  (TNF- $\alpha$ ) exerts its effect on cells by binding to two distinct receptors, TNF-R1 (55 kDa) and TNF-R2 (75 kDa) (33). These two receptors bind TNF- $\alpha$  with high affinity but differ in their intracellular domains and mediate distinct cellular responses (33). The type I receptor is thought to confer cytotoxic signals and mediate cell adhesion, whereas the type II receptor has been linked to mitogenic responses (32, 34). Both receptor types have been implicated in TNF- $\alpha$ -induced apoptosis (32). Signaling pathways initiated by TNF- $\alpha$  include the activation of protein kinase C (PKC), phospholipase A<sub>2</sub> (PLA<sub>2</sub>), phosphatidylcholine phospholipase C, and plasma mem-

brane-associated neutral and endosomal acidic sphingomyelinase (9, 27, 28, 35). TNF- $\alpha$  has also been shown to induce serine-threonine and tyrosine phosphorylation of various cellular proteins (11).

In cultured endothelial cells, the major effect of TNF- $\alpha$  is the stimulation of the transcription of genes encoding the endothelial cell adhesion molecules E-selectin (endothelial-leukocyte adhesion molecule 1, or ELAM-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (4). The promoter regions of these genes contain nuclear factor (NF)- $\kappa$ B binding sites, which have been implicated in the TNF- $\alpha$ -mediated induction of these genes (5). Increased tissue levels of TNF- $\alpha$  and overexpression of these adhesion molecules may play an important role in the pathogenesis of some diseases, such as the proliferative phase of diabetic retinopathy (18). TNF- $\alpha$  reduces proteoglycan synthesis, which may impair endothelial barrier function (10), and induces production of reactive oxygen species, which could increase oxidant-induced injury of the endothelium, especially in diseases such as diabetes in which antioxidant pathways are compromised (8). In these studies, we present another effect of TNF- $\alpha$  on the endothelium. The accumulation of *myo*-inositol and its subsequent incorporation into phosphoinositides are significantly decreased by TNF- $\alpha$  in large-vessel endothelial cells. The effect of TNF- $\alpha$  persists over an extended period of time and is preceded by a downregulation of Na<sup>+</sup>-*myo*-inositol cotransporter (SMIT) mRNA levels. Because the normal metabolism of *myo*-inositol is critical to maintaining phosphoinositide synthesis and because several signal transduction pathways utilize phosphoinositides, including the phosphatidylinositol cycle and phosphatidylinositol 3-kinase, TNF- $\alpha$  may alter endothelial cell function by reducing *myo*-inositol metabolism.

## MATERIALS AND METHODS

**Materials.** Chemicals, neutral red, interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , and IL-2, genistein, orthovanadate, *N*-oleoylethanolamine, staurosporin, 4-bromophenacyl bromide, sphingosine, pyrrolidinedithiocarbamate, 7-amino-1-chloro-3-tosylamido-2-hepatanone (TLCK) C<sub>2</sub>-ceramide, and lipopolysaccharide (LPS) were from Sigma (St. Louis, MO) unless otherwise noted. Insulin-like growth factor I (IGF-I) was from Intergen (Purchase, NY). IL-6 was from Bachem Bioscience (King of Prussia, PA). Anti-p50, anti-p65, and anti-cAMP response

element binding protein (CREB) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chloroform, methanol, isoamyl alcohol, ethanol, Corning 75-cm<sup>2</sup> flasks, and Falcon six-well plates were from Fisher Scientific (Fair Lawn, NJ). Sodium dodecyl sulfate was from British Drug House (Poole, UK). Ethidium bromide and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were from Boehringer Mannheim (Indianapolis, IN). Pyridine, trimethylchlorosilane, and hexamethyldisilazane were from Pierce (Rockford, IL). Transcription buffer, dithiothreitol, RNasin, ATP, CTP, UTP, GTP, T7 RNA polymerase, and deoxyribonuclease were from Promega (Madison, WI). The 18S antisense ribosomal RNA probe and  $\beta$ -actin mRNA probe were from Ambion (Austin, TX). Myo-[2-<sup>3</sup>H]inositol, [methyl-<sup>3</sup>H]choline chloride, L-[4,5-<sup>3</sup>H]leucine, and [<sup>32</sup>P]UTP were from Amersham (Arlington Heights, IL). Safety-Solve, cesium chloride, and scintillation vials were from Research Products International (Mount Prospect, IL). Recombinant human TNF- $\alpha$  and transforming growth factor- $\beta$  (TGF- $\beta$ ) were from Research & Development Systems (Minneapolis, MN). Wortmannin, rapamycin, and calphostin C were from Calbiochem (La Jolla, CA). PD-98059 was a kind gift from Dr. Alan Saltiel, Department of Signal Transduction, Parke-Davis Pharmaceutical Division (Ann Arbor, MI). SB-203580 was a kind gift from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Cell culture medium was obtained from the Diabetes-Endocrinology Research Center Cell Biology core, University of Iowa (Iowa City, IA). Synthetic oligonucleotides used for gel mobility shift assays were provided by Genosys (Woodlands, TX) through the Diabetes-Endocrinology Research Center Molecular Biology Core, University of Iowa.

**Cell culture.** Murine MB114 cerebral microvessel endothelial (CME) cells were obtained from Dr. Steven Moore, University of Iowa. The cells were grown in M199 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and basal medium Eagle amino acid and vitamin solutions. Bovine aortic endothelial (BAE) cells originated from freshly slaughtered steers and were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. Bovine pulmonary artery (PA) endothelial cells were kindly provided by Dr. Robert Bar, University of Iowa, and were grown in the same medium as described above for the BAE cells. Endothelial cells from bovine periaortic adipose tissue and coronary artery were kindly provided by Dr. Robert Bar and were grown in the same medium as described above for CME cells. Murine neuroblastoma cells, NB41A3, were obtained from the American Type Culture Collection and grown in Ham's F-10 medium supplemented with 2.5% heat-inactivated fetal bovine serum, 15% horse serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. Human skin fibroblasts were kindly provided by Dr. Robert Spanheimer, University of Iowa, and were grown in minimal essential medium with Earle's salts supplemented with 15% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 294  $\mu$ g/ml glutamine, and basal medium Eagle amino acid and vitamin solutions. Human Hep G2 hepatoma cells were kindly provided by Dr. Jeffrey Fields, University of Iowa, and grown in Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. Murine cortical collecting duct and rat inner medullary collecting duct cells were kindly provided by Dr. John Stokes, University of Iowa, and were grown in Dulbecco's modified Eagle's medium-F-12 supple-

mented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. Rat C6 glioma cells and rat smooth muscle cells were kindly provided by Dr. Arthur Spector, University of Iowa. C6 glioma cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. Rat smooth muscle cells were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 294  $\mu$ g/ml glutamine. All cells were propagated in Corning 75-cm<sup>2</sup> flasks in an incubator maintained at 37°C with 5% CO<sub>2</sub> in humidified air. Cells were passed weekly at a dilution ranging from 1:10 to 1:20 and were fed three times per week by replacing the medium. Endothelial cells between passages 6 and 15 were used in these studies. For myo-inositol accumulation studies, the cells were seeded onto Falcon six-well cluster plates and assays were conducted in triplicate when the cells reached confluency. For other studies, cells were seeded in either six-well plates or 25-, 75-, or 150-cm<sup>2</sup> flasks. All studies were conducted when cells reached confluency.

**Myo-inositol accumulation and cellular myo-inositol determination.** For myo-inositol accumulation determination, cells were incubated in M199 serum-free medium containing 0.5% bovine serum albumin for 1–24 h in the absence or presence of 0.01–10 ng/ml TNF- $\alpha$ . Afterwards, the cells were washed with serum-free medium and then incubated for 10–60 min in 2 ml of serum-free medium containing myo-[2-<sup>3</sup>H]inositol. The myo-inositol concentration of the serum-free medium was 11.4  $\mu$ M. After the incubation, cells were quickly washed two times with ice-cold 10 mM HEPES buffer, pH 7.4, containing 128 mM NaCl, 5.2 mM KCl, 2.1 mM CaCl<sub>2</sub>, 2.9 mM MgSO<sub>4</sub>, and 5 mM glucose and were collected by scraping the cells in 1.5 ml water. The cell suspension was sonicated for 5 s, and samples were taken to determine protein content and myo-inositol accumulation. Myo-inositol accumulation was determined by taking duplicate aliquots of the cell suspension and measuring the radioactivity present using a Beckman LS8100 (Fullerton, CA) liquid scintillation counter. Protein content was determined in duplicate aliquots of the cell suspension using a modification of the Lowry method (17). For many of these studies, myo-inositol accumulation was determined after a 1-h incubation. In these studies, the term "myo-inositol accumulation" represents the cellular accumulation of radioactivity derived from myo-[2-<sup>3</sup>H]inositol during the incubation and does not represent the initial rate of myo-inositol transport by the cells, nor does it account for any myo-[2-<sup>3</sup>H]inositol taken up by the cells and then secreted during the 1-h incubation period. TNF- $\alpha$  did not cause an increase in the turnover of radioactivity after metabolic labeling of the cells with myo-[2-<sup>3</sup>H]inositol. Therefore, secretion of myo-[2-<sup>3</sup>H]inositol during the 1-h incubation period likely has little effect on these results. Besides turnover, the uptake of myo-inositol over a 1-h period could be influenced by other factors such as changes in intracellular metabolism; therefore, myo-inositol uptake studies using shorter incubation periods as well as kinetic analysis of high-affinity myo-inositol transport were conducted to validate the results obtained from studies using a 1-h incubation period.

Besides TNF- $\alpha$ , the effect of other cytokines and growth factors on myo-inositol accumulation by cultured endothelial cells was examined. For these studies, the cells were incubated in serum-free medium for 16 h in the absence or presence of IL-1 $\alpha$ , IL-1 $\beta$ , IGF-I, IL-2, IL-6, or TGF- $\beta$  at the concentrations indicated in the legend of Fig. 4. Afterwards, the accumulation of myo-inositol was determined as described above (see *Myo-inositol accumulation and cellular*

*myo-inositol determination*). To examine the effect of C<sub>2</sub>-ceramide, sphingosine, or LPS on *myo*-inositol accumulation, cells were incubated in serum-free medium in the absence or presence of 10–100  $\mu$ M C<sub>2</sub>-ceramide, sphingosine (50  $\mu$ M), or 1  $\mu$ g/ml LPS for 16 h, and, afterwards, the accumulation of *myo*-inositol was examined as described above. In studies examining the effect of inhibitors on the TNF- $\alpha$ -mediated decrease of *myo*-inositol accumulation, the cells were preincubated for 1 h in serum-free medium in the absence or presence of each inhibitor at the concentration indicated in Table 2. Afterwards, 5 ng/ml TNF- $\alpha$  was added, and the incubation was continued for an additional 15 h. The accumulation of *myo*-inositol was then determined as described above. To examine the combined effect of TNF- $\alpha$  and hyperosmolarity on *myo*-inositol accumulation, cells were preincubated in serum-free medium containing TNF- $\alpha$  (5 ng/ml) for 1 h before the addition of 150 mM raffinose [to induce hyperosmolarity (36)]. Other cells were incubated in serum-free medium alone or this medium containing TNF- $\alpha$  (5 ng/ml) or 150 mM raffinose. After 24 h, the cells were washed, and *myo*-inositol accumulation was determined by incubating the cells for 1 h in osmotically matched HEPES buffer containing 10  $\mu$ M *myo*-[2-<sup>3</sup>H]inositol. To examine *myo*-inositol accumulation and incorporation into phosphoinositides, cells were incubated in serum-free medium containing 5 ng/ml TNF- $\alpha$  for 16 h followed by an incubation in serum-free medium containing *myo*-[2-<sup>3</sup>H]inositol for 6 h. Afterwards, samples were taken to determine total *myo*-inositol accumulation and protein content as described above, as well as the amount of *myo*-[2-<sup>3</sup>H]inositol incorporated into phosphoinositides. For the latter determination, an aliquot of the cell suspension (0.5 ml) was extracted with 10 ml of chloroform-methanol-HCl (2:1:0.015), and the lipid (organic) phase containing the phospholipid fraction was separated from the aqueous fraction by the addition of 2 ml of acidic saline and by mixing. The lower phase was collected in a scintillation vial, and the chloroform was evaporated in a fume hood followed by the addition of scintillation solution and determination of the amount of radioactivity present in the lipid fraction. *Myo*-inositol accumulation and incorporation into phosphoinositides were calculated as nanomoles per milligrams of cell protein. To determine the effect of TNF- $\alpha$  on Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent *myo*-inositol uptake, cells were incubated in HEPES buffer. For Na<sup>+</sup>-independent *myo*-inositol uptake determination, the cells were washed and incubated in this buffer containing choline chloride in place of NaCl. Kinetic parameters for high-affinity *myo*-inositol transport were determined by incubating cells for 5 min in HEPES buffer with or without NaCl and containing 5–100  $\mu$ M *myo*-[2-<sup>3</sup>H]inositol, as previously described (37). Uptake of *myo*-inositol occurring in the absence of NaCl was subtracted from uptake in the presence of NaCl before determining the apparent (indicated by the prime)  $K_m$  and maximal velocity ( $V_{max}$ ) for high-affinity *myo*-inositol transport (37). For the latter two studies, cells were treated with or without TNF- $\alpha$  before the examination of *myo*-inositol uptake.

To examine the effect of TNF- $\alpha$  on choline accumulation and incorporation into phospholipid and leucine accumulation and incorporation into cell proteins, BAE cells were incubated in serum-free medium in the absence or presence of TNF- $\alpha$  (5 ng/ml) for 16 h. Afterwards, the cells were incubated for an additional 6 h in serum-free medium containing either [methyl-<sup>3</sup>H]choline chloride or L-[4,5-<sup>3</sup>H]leucine. The total accumulation of choline or leucine was determined as described for *myo*-inositol. Choline incorporation into phospholipid was determined as described, and leucine incorporation into cell protein was determined by perchloric acid (PCA)

precipitating the protein from an aliquot of the cell suspension followed by centrifugation and washing of the pellet with an additional 10% PCA. Afterwards, the amount of radioactivity in the cell pellet was determined. For some studies, the cells were incubated with either [methyl-<sup>3</sup>H]choline chloride or L-[4,5-<sup>3</sup>H]leucine for 15–60 min to examine the effect of TNF- $\alpha$  on choline or leucine accumulation after a shorter incubation period. As indicated above, these studies did not examine the effect of TNF- $\alpha$  on the initial rate of choline or leucine uptake.

To determine the effect of TNF- $\alpha$  on intracellular *myo*-inositol content, cells were grown in 25-cm<sup>2</sup> flasks to confluency and then incubated in serum-free medium in the absence or presence of TNF- $\alpha$  (5 ng/ml) for 24 h. Afterwards, the cells were washed with glucose-free HEPES buffer, collected in water, and sonicated. Aliquots of the cell suspension were taken for protein determination and derivatization as described previously (36). The derivatized samples were then chromatographed on a temperature-programmed Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) interfaced with a model HP3390A integrator. The initial temperature of 180°C was maintained for 2 min and was then increased at 4°C/min to a final temperature of 225°C, which was maintained for 5 min. The column consisted of 3% SE-30 on Supelcoport (Supelco, Bellefonte, PA). An authentic *myo*-inositol standard was run to verify its elution time, and methyl  $\alpha$ -D-mannopyranoside was added as an internal standard (36). The *myo*-inositol content was calculated as nanomoles per milligram of cell protein.

*Neutral red assay*. A neutral red assay was used to measure cytopathogenicity (7). BAE and murine CME cells were incubated in serum-free medium in the absence or presence of TNF- $\alpha$  (5 ng/ml) for 16 h. Afterwards, 3  $\mu$ l of 1% neutral red were added, and the cells were incubated for an additional 2 h at 37°C. The medium was then removed by aspiration, and the cell monolayer was washed three times with HEPES buffer. The neutral red was extracted with 1 ml of 50% ethanol and 50 mM sodium citrate, pH 4.2, and absorbance was measured at 540 nm. The data were calculated as percent of control.

*Ceramide assay*. After treatment of BAE or murine CME cells with TNF- $\alpha$  for 5–45 min, the cells were extracted with chloroform-methanol-HCl as described. To determine ceramide levels, the lipid fraction was incubated with diacylglycerol kinase as described previously (19). The major lipid products of the phosphorylation reaction were phosphatidic acid (from diacylglycerol) and ceramide 1-phosphate (from ceramide). These products were completely resolved by thin-layer chromatography, using chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1) as solvent, and were visualized by autoradiography. The data were calculated as picomoles of ceramide per nanomoles of lipid phosphorus, based on a ceramide standard curve generated with C<sub>6</sub>-ceramide.

*Electrophoretic mobility shift assay*. Confluent cells were treated with 5 ng/ml TNF- $\alpha$  or hyperosmolarity in serum-free medium for 15 or 30 min. For some studies, cells were pretreated with genistein (50  $\mu$ M), TLCK (50  $\mu$ M), calphostin C (1  $\mu$ M), or pyrrolidinedithiocarbamate (100  $\mu$ M) for 1 h before the addition of TNF- $\alpha$ , and the incubations were then continued for an additional 30 min. Afterwards, the cells were washed and harvested with phosphate-buffered saline (PBS) at 4°C and with slow-speed centrifugation. The cells were resuspended in 4 ml of buffer A (10.0 mM HEPES, pH 8.0, 1.5 mM MgCl<sub>2</sub>, 10.0 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 0.1% Nonidet P-40, 1  $\mu$ g/ml each pepstatin, antipain, chymostatin, and aprotinin, 0.1  $\mu$ g/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) and left on ice for 5 min. The crude nuclear pellet was then collected by microcentrifugation for 2 min at 4°C. Afterwards, the pellet was quickly

washed with *buffer A* and resuspended in *buffer B* (20 mM HEPES, pH 8.0, 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1  $\mu$ g/ml each pepstatin, antipain, chymostatin, and aprotinin, and 0.1  $\mu$ g/ml leupeptin). The nuclei were sonicated for 10 s at 4°C and clarified by microcentrifugation. Protein concentration of the extract was determined using the method of Lowry (17), and the extract was stored at -70°C. For gel mobility shift assays, annealed oligonucleotides containing the consensus sequence for NF- $\kappa$ B (5'-TTTCGCGGGGACTTTCCCGCGC-3'; 5'-TTTGC GCGCGAA-ATCCCCGCG-3'), mutant NF- $\kappa$ B (5'-TTTCGCGCGGACA-TTCCCGCGC-3'; 5'-TTTGC GCGGGAATGTCCGCGCG-3'), and the E-box of the adenovirus major late transcription factor promoter (5'-ATAGGTGTAGGCCACGTGACCGGGTGT-3'; 5'-ACACCGGTCACGTG-3') were radiolabeled with [ $\gamma$ - $^{32}$ P]dATP and unlabeled dGTP, dCTP, and dTTP using Klenow DNA polymerase or *Taq* polymerase and gel purified. Twenty micrograms of nuclear extract were preincubated for 10 min at 25°C with 1  $\mu$ g poly(dIdC)·poly(dIdC) under ionic conditions. For a nonspecific control, a 10- or 50-fold excess of unlabeled oligonucleotide was included in some incubations. Radiolabeled probe (5  $\times$  10<sup>4</sup> counts/min, ~2 ng) was added to each 20- $\mu$ l reaction and incubated for 15 min at 37°C. Samples were analyzed on a 5% nondenaturing polyacrylamide gel in 0.5  $\times$  tris(hydroxymethyl)aminomethane-borate-EDTA (45 mM tris(hydroxymethyl)aminomethane-borate, 1 mM EDTA, pH 8.0) and electrophoresed at 115 V for 3 h at 25°C. Gels were then dried, and autoradiographs were exposed for the appropriate period at -80°C with intensifying screens. For supershift analysis, nuclear extracts from TNF- $\alpha$ -treated BAE or PA endothelial cells were preincubated for 15 min at room temperature with 1  $\mu$ g of anti-p50, anti-p65, or anti-CREB rabbit polyclonal antibodies. Afterwards, the radiolabeled oligonucleotides were added and examined as described above.

**RNA isolation and gel electrophoresis.** RNA was prepared using the guanidine isothiocyanate-cesium chloride method. RNA was quantitated by measuring the absorbance at 260 nm, and the integrity of the RNA and accuracy of quantification were confirmed by size separating the RNA by denaturing gel electrophoresis and comparing the intensity of the 18S and 28S ribosomal RNA bands after ethidium bromide staining of the gel.

**Quantification of SMIT mRNA levels.** SMIT mRNA levels in CME cells were quantified using a solution hybridization-ribonuclease (RNase) protection assay as previously described (36). Briefly,  $^{32}$ P-labeled antisense SMIT mRNAs were transcribed, using T7 RNA polymerase and a SMIT cDNA construct in pGEM-3Zf(+) that had been linearized with *Hind* III. Antisense SMIT mRNAs were then incubated at 45°C in 75% formamide-0.4 M NaCl with 20  $\mu$ g of total RNA. After a 16-h incubation, the samples were digested with RNases A and T<sub>1</sub>. The protected double-stranded hybrids were collected by ethanol precipitation and electrophoresed through an 8% polyacrylamide-8 M urea denaturing gel. To confirm equal loading of the gel, 18S ribosomal RNA was determined simultaneously with the use of a commercially available 18S antisense control template that binds to an 80-nucleotide fragment from a conserved region of the 18S ribosomal RNA. The antisense 18S RNAs were generated per the manufacturer's instructions using T7 polymerase. A sufficient quantity of each of the antisense SMIT mRNA and 18S rRNA probes was added to each sample to ensure the presence of an excess of labeled antisense RNA. These data were obtained empirically by conducting an RNase protection assay using 20–80  $\mu$ g of total RNA and a constant amount of

antisense SMIT mRNA or 18S rRNA probe. When the assay was conducted, with use of an amount of antisense RNA that was similar to that used in the RNase protection assays, a linear response was seen when up to 80  $\mu$ g of total RNA was used in the assay. This represents four times the amount of total RNA that was used in the RNase protection assays reported in this study. SMIT mRNAs were represented as a single band on the autoradiogram of the gel, with the intensity of the band being proportional to the SMIT mRNA level in the sample. SMIT mRNA levels were quantified by scanning densitometry of the autoradiogram using a GS 300 transmittance-reflectance scanning densitometer (Hoefer, San Francisco, CA) interfaced with a model HP 3396A (Hewlett-Packard) integrator and standardized to the intensity of the 18S rRNA band.

**Northern blot analysis.** To determine BAE cell SMIT mRNA levels, Northern blot analyses were conducted as described previously (38). Briefly, RNA was separated by electrophoresis using a 1% agarose-formaldehyde denaturing gel as described and blotted to a nylon membrane for 16 h in 20 $\times$  saline sodium citrate. After blotting, the nylon membrane was baked at 80°C for 1.5 h under vacuum. The blot was then subjected to hybridization with a  $^{32}$ P-labeled 626-base pair SMIT cDNA that was generated using a random primed DNA labeling kit (Boehringer Mannheim) or a  $^{32}$ P-labeled  $\beta$ -actin genomic DNA probe, according to previously described procedures (38). After hybridization, the blot was subjected to a stringent wash procedure (38). Afterwards, the blot was subjected to autoradiography, and the level of the SMIT mRNA was quantified by scanning densitometry and standardized to the intensity of the  $\beta$ -actin mRNA.

**Data analysis.** Data for *myo*-inositol accumulation are reported as nanomoles per milligrams cell protein or as percent of control. Statistical comparisons for significance were performed using an unpaired Student's *t*-test or Dunnett's analysis at a *P* value of 0.05.

## RESULTS

**Effect of TNF- $\alpha$  on *myo*-inositol accumulation.** Previously we had shown that hyperosmolarity increased *myo*-inositol accumulation in cultured endothelial cells and that the increase in accumulation was preceded by an increase in SMIT mRNA levels (36). From these studies, we concluded that exposing cultured endothelial cells to a hyperosmotic medium increased SMIT gene and protein expression, resulting in an increase in *myo*-inositol accumulation. The signal transduction pathway thought to be responsible for mediating some of the effects of the hyperosmotic response is the mitogen-activated protein (MAP) kinase family of protein kinases. Exposing mammalian cells to hyperosmolarity has been shown to activate multiple members of the MAP kinase family, including the extracellular signal-regulated kinases (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK) and p38 kinase (14). Because TNF- $\alpha$  also activates ERK, JNK, and p38 kinase, we examined the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation (14, 24). Shown in Fig. 1 is the effect of TNF- $\alpha$  (10 ng/ml) on *myo*-inositol accumulation by a variety of cultured mammalian cells. Cells were incubated in serum-free medium in the absence or presence of TNF- $\alpha$  for 16 h. Afterwards, *myo*-inositol accumulation was determined by incubating the cells for 1 h in fresh serum-free medium containing *myo*-[2- $^3$ H]inosi-

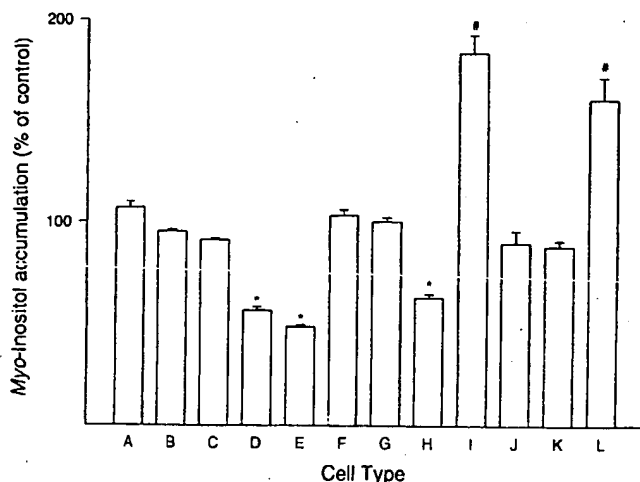


Fig. 1. Effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on *myo*-inositol accumulation in cultured mammalian cells. Cells were grown in 6-well plates until confluency and then incubated for 16 h in serum-free medium containing 0.5% bovine serum albumin in the absence or presence of 10 ng/ml TNF- $\alpha$ . Afterwards, cells were washed and incubated for 1 h in serum-free medium containing 11.4  $\mu$ M *myo*-[2- $^3$ H]inositol, and *myo*-inositol accumulation was determined as described (see MATERIALS AND METHODS). Accumulation of *myo*-inositol is expressed as a percentage of *myo*-inositol accumulation in respective control cells, which were not exposed to TNF- $\alpha$ , and is defined here as 100%. Cells used in these studies and basal accumulation of *myo*-inositol in 1 h (expressed as nmol/mg protein) by each were as follows: A, human skin fibroblasts,  $1.07 \pm 0.09$ ; B, rat smooth muscle cells,  $0.95 \pm 0.04$ ; C, human Hep G2 cells,  $0.91 \pm 0.04$ ; D, bovine aorta endothelial (BAE) cells,  $0.56 \pm 0.06$ ; E, bovine pulmonary artery (PA) endothelial cells,  $0.48 \pm 0.03$ ; F, rat C6 glioma cells,  $1.03 \pm 0.09$ ; G, murine neuroblastoma cells,  $1.00 \pm 0.09$ ; H, murine cerebral microvessel endothelial (CME) cells,  $0.62 \pm 0.06$ ; I, bovine periaortic adipose microvessel endothelial cells,  $1.83 \pm 0.27$ ; J, murine cortical collecting duct cells,  $0.89 \pm 0.18$ ; K, rat inner medullary collecting duct cells,  $0.87 \pm 0.09$ ; and L, bovine coronary artery endothelial cells,  $0.21 \pm 0.07$ . Each value is mean  $\pm$  SE of 9 separate experiments. \*Significant decrease ( $P < 0.05$ ) compared with control; # significant increase ( $P < 0.05$ ) compared with control.

tol. In contrast to the effect of hyperosmolarity, TNF- $\alpha$  significantly reduced *myo*-inositol accumulation in BAE cells (column D), bovine PA endothelial cells (column E), and murine CME cells (column H). In contrast, TNF- $\alpha$  did not affect *myo*-inositol accumulation by human skin fibroblasts (column A), rat smooth muscle cells (column B), human Hep G2 cells (column C), rat C6 glioma cells (column F), murine neuroblastoma cells (column G), murine cortical collecting duct cells (column J), or rat inner medullary collecting duct cells (column K). *Myo*-inositol accumulation was significantly increased by TNF- $\alpha$  in bovine microvessel endothelial cells derived from periaortic adipose tissue and bovine coronary artery endothelial cells (columns I and L, respectively). The studies reported here were conducted using serum-free medium; however, when cells were incubated with or without TNF- $\alpha$  in their normal culture medium followed by a determination of *myo*-inositol accumulation, similar results were obtained (data not shown).

The concentration curve and time course dependence for the TNF- $\alpha$ -mediated decrease in *myo*-inositol accumulation were examined in murine CME, BAE, and

bovine PA endothelial cells (Fig. 2). These studies demonstrated that the effect of TNF- $\alpha$  on *myo*-inositol accumulation was dose dependent and that a maximum inhibition of *myo*-inositol accumulation was achieved with 5 ng/ml TNF- $\alpha$ . The response to TNF- $\alpha$  was also time dependent. A 6-h incubation with 5 ng/ml TNF- $\alpha$  was sufficient to cause a maximal inhibition of *myo*-inositol accumulation in murine CME and bovine PA endothelial cells, whereas a 24-h incubation was necessary for BAE cells. This variability in time dependence is likely due to the different turnover rates of the SMIT protein in each cell type. To validate the results obtained using a 1-h incubation period with *myo*-[2- $^3$ H]inositol, we examined the effect of TNF- $\alpha$  on *myo*-inositol uptake by murine CME, bovine PA endothelial, and BAE cells after a 10–60 min incubation (Fig. 3). Even though *myo*-inositol accumulation by the cells over this time period was not precisely linear, the results clearly demonstrate that *myo*-inositol accumulation is significantly decreased by TNF- $\alpha$ .

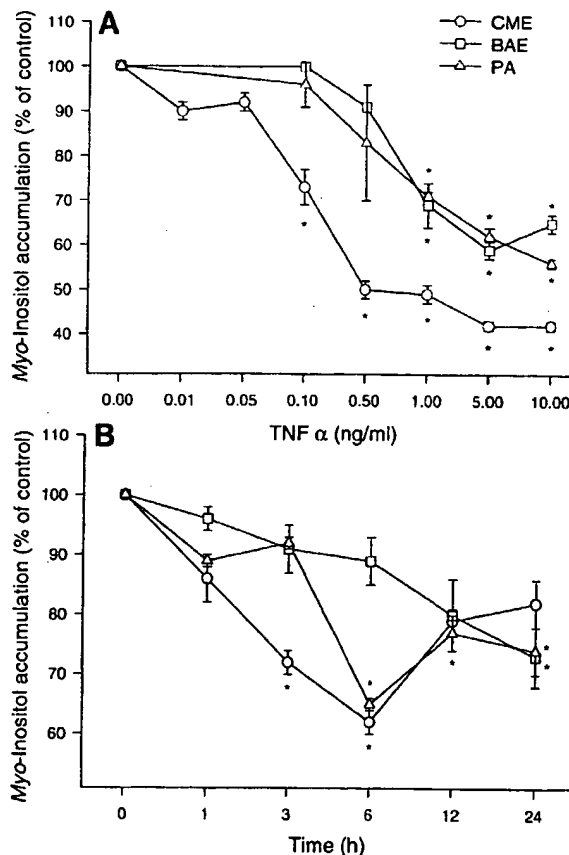


Fig. 2. Concentration- and time-dependent decrease in *myo*-inositol accumulation by TNF- $\alpha$ . Murine CME, BAE, and bovine PA endothelial cells were grown in 6-well plates and then exposed to various concentrations of TNF- $\alpha$  (0–10 ng/ml) (A) or to 5 ng/ml TNF- $\alpha$  for 1–24 h (B). *Myo*-inositol accumulation was then determined as described in MATERIALS AND METHODS. *Myo*-inositol accumulation is expressed as percentage of control, which is defined as 100%. Basal *myo*-inositol accumulation by murine CME, BAE, and bovine PA endothelial cells after a 1-h incubation was  $0.48 \pm 0.02$ ,  $0.31 \pm 0.03$ , and  $0.32 \pm 0.03$  nmol/mg protein, respectively. Each value is the mean  $\pm$  SE of 9 separate experiments. \**Myo*-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with control.



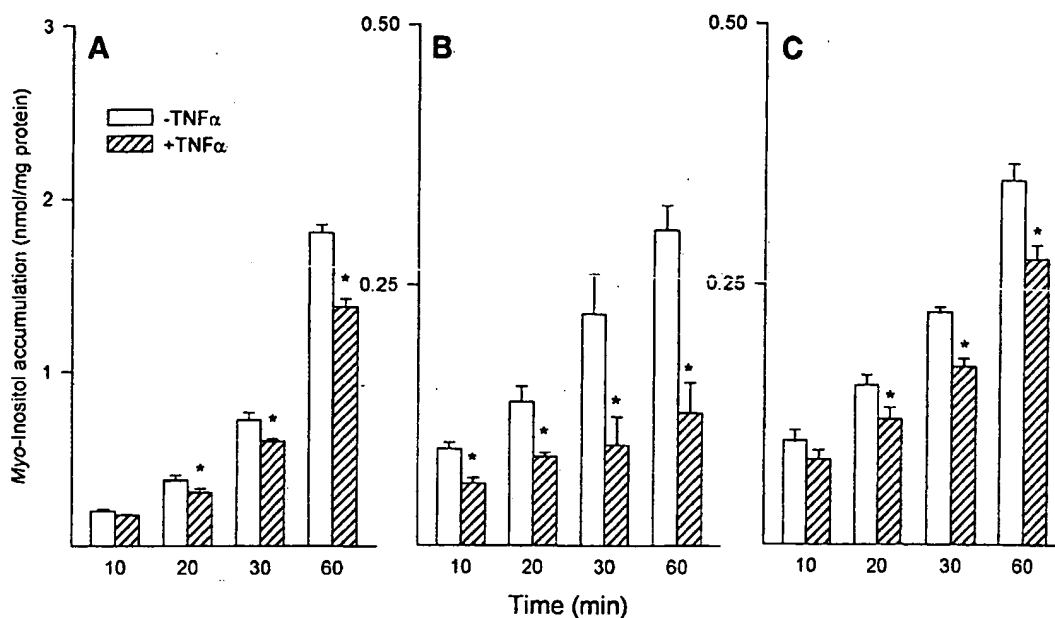


Fig. 3. Effect of TNF- $\alpha$  on myo-inositol accumulation. Murine CME (A,  $n = 6$ ), bovine PA endothelial (B,  $n = 6$ ), and BAE (C,  $n = 9$ ) cells were grown in 6-well plates to near confluency and then incubated for 16 h in serum-free medium in absence (-) or presence (+) of TNF- $\alpha$  (5 ng/ml). Myo-inositol accumulation, expressed as nmol/mg protein, was then determined in triplicate after a 10- to 60-min incubation as described. \* Myo-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with control.

To determine the specificity of the effect of TNF- $\alpha$  on myo-inositol accumulation, we examined the effect of growth factors and other cytokines on myo-inositol accumulation by bovine PA endothelial, BAE, and murine CME cells (Fig. 4). After a 16-h incubation in

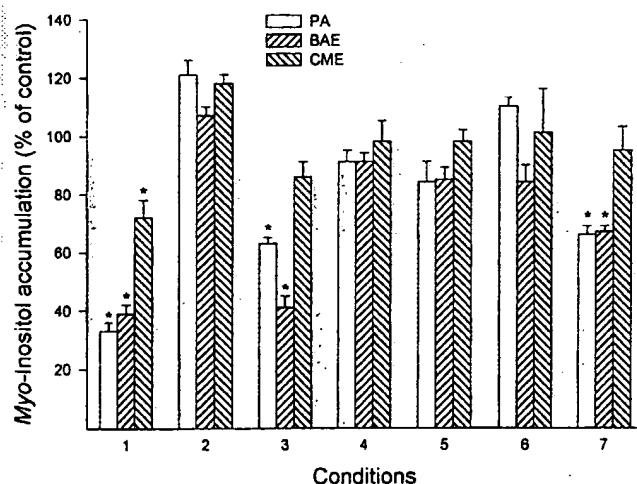


Fig. 4. Effect of cytokines and growth factors on myo-inositol accumulation. Bovine PA endothelial, BAE, or murine CME cells were grown in 6-well plates to near confluency and then incubated for 16 h in serum-free medium in absence or presence of TNF- $\alpha$  (5 ng/ml, condition 1), insulin-like growth factor I (50 ng/ml, condition 2), interleukin (IL)-1 $\beta$  (10 ng/ml, condition 3), IL-1 $\alpha$  (10 ng/ml, condition 4), IL-2 (10 ng/ml, condition 5), IL-6 (10 ng/ml, condition 6), or transforming growth factor- $\beta$  (5 ng/ml, condition 7). Myo-inositol accumulation was then determined as described in MATERIALS AND METHODS. Myo-inositol accumulation is expressed as percentage of control, which is defined as 100%. Each value is mean  $\pm$  SE of 6 separate experiments. \* Myo-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with control.

serum-free medium, TNF- $\alpha$  (5 ng/ml, condition 1), IL-1 $\beta$  (10 ng/ml, condition 3) and TGF- $\beta$  (5 ng/ml, condition 7) significantly decreased myo-inositol accumulation by bovine PA endothelial and BAE cells. Myo-inositol accumulation by murine CME cells was reduced by only TNF- $\alpha$ . In contrast, IGF-1 (50 ng/ml, condition 2), IL-1 $\alpha$  (10 ng/ml, condition 4), IL-2 (10 ng/ml, condition 5), and IL-6 (10 ng/ml, condition 6) did not alter myo-inositol accumulation.

Determination of the effect of TNF- $\alpha$  on Na<sup>+</sup>-dependent myo-inositol uptake by BAE cells showed that TNF- $\alpha$  had no effect on Na<sup>+</sup>-independent myo-inositol uptake. Na<sup>+</sup>-independent and Na<sup>+</sup>-dependent myo-inositol uptakes by BAE cells after a 10-min incubation period in buffer containing 10  $\mu$ M myo-inositol were  $0.017 \pm 0.006$  and  $0.143 \pm 0.007$  nmol/mg protein, respectively (mean  $\pm$  SE,  $n = 6$ ). After treatment for 16 h with 5 ng/ml TNF- $\alpha$ , Na<sup>+</sup>-independent and Na<sup>+</sup>-dependent myo-inositol uptakes were  $0.014 \pm 0.002$  and  $0.071 \pm 0.007$  nmol/mg protein ( $P < 0.05$ ), respectively. Similar results were obtained with bovine PA endothelial and CME cells (data not shown). Because the above studies (Figs. 1–4) did not address the effect of TNF- $\alpha$  on the initial rate of myo-inositol uptake, kinetic analysis of the effect of TNF- $\alpha$  on high-affinity myo-inositol transport was conducted. These studies demonstrated that TNF- $\alpha$  caused a significant decrease in  $V'_{\max}$  with no change in  $K'_m$ .  $K'_m$  for high-affinity myo-inositol transport by BAE cells treated with and without TNF- $\alpha$  for 16 h was  $60.3 \pm 4.8$  and  $65.2 \pm 12.0$   $\mu$ M, respectively, and  $V'_{\max}$  was  $210.7 \pm 12.5$  and  $143.8 \pm 15.2$  pmol  $\cdot$  mg protein<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, respectively (mean  $\pm$  SE,  $n = 3$ ) ( $P < 0.05$ ). Studies conducted with bovine PA endothelial cells gave similar results (data not shown).

Because phosphoinositide production is linked to *myo*-inositol uptake, we examined the effect of TNF- $\alpha$  on *myo*-inositol accumulation and incorporation into phosphoinositides in BAE cells. After a 6-h incubation, *myo*-inositol accumulation and incorporation into phosphoinositides were decreased by 60 and 40%, respectively, in cells treated with 5 ng/ml TNF- $\alpha$  for 16 h (Table 1). The free *myo*-inositol content was also significantly decreased after a 24-h incubation in serum-free medium containing 5 ng/ml TNF- $\alpha$ .

To determine whether the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation is due to an increase in *myo*-inositol efflux, BAE cells were prelabeled with *myo*-[2- $^3$ H]inositol for 16 h and were then washed and incubated in the absence or presence of TNF- $\alpha$  for up to 24 h. At 1, 3, 6, and 24 h, samples were collected to determine the amount of *myo*-[2- $^3$ H]inositol remaining in the cells and appearing in the medium. Results from this study demonstrated that *myo*-inositol efflux was not increased by TNF- $\alpha$  and that >60% of the *myo*-[2- $^3$ H]inositol taken up by the cells during the pulse period remained cell associated after 24 h (data not shown).

**Recovery of *myo*-inositol accumulation.** Data in Fig. 5 show the recovery of *myo*-inositol accumulation by cells preincubated in serum-free medium containing TNF- $\alpha$  (5 ng/ml) for 16 h followed by a 24-h incubation in serum-free medium alone for murine CME, BAE, and bovine PA endothelial cells. *Myo*-inositol accumulation was significantly improved in cells exposed to TNF- $\alpha$  after a 24-h washout or recovery period compared with TNF- $\alpha$ -treated cells; however, *myo*-inositol accumulation by the reverted cells remained significantly decreased compared with control cells. The recovery ranged from 20 to 75% after the 24-h incubation.

**Effect of TNF- $\alpha$  on choline and leucine accumulation.** To determine whether the effect of TNF- $\alpha$  on *myo*-inositol accumulation was specific or whether TNF- $\alpha$  affected the accumulation of other molecules required for membrane or protein synthesis, we examined the effect of TNF- $\alpha$  on choline accumulation and incorporation into phospholipid and L-leucine accumulation and

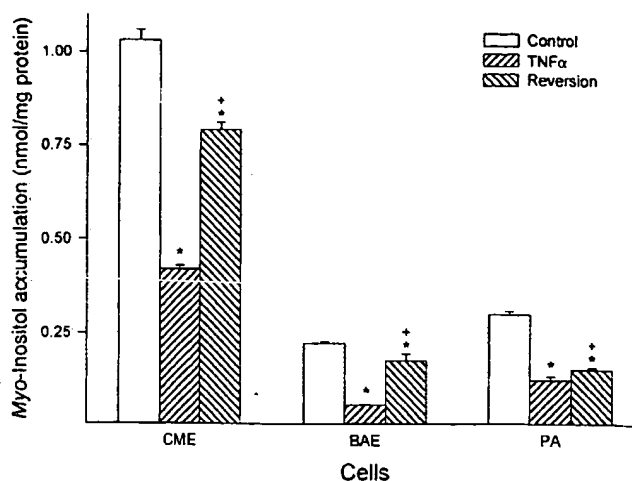


Fig. 5. Reversion of effect of TNF- $\alpha$  on *myo*-inositol accumulation by murine CME, BAE, and bovine PA endothelial cells. Cells were grown in 6-well plates and then incubated for 16 h in serum-free medium (control) or same medium containing 5 ng/ml TNF- $\alpha$ . Afterwards, some cells incubated in medium containing TNF- $\alpha$  were washed and then incubated for 24 h in normal serum-free medium. *Myo*-inositol accumulation was then determined by incubating cells for 1 h in serum-free medium containing *myo*-[2- $^3$ H]inositol. *Myo*-inositol accumulation is expressed as nmol/mg protein. Each value is mean  $\pm$  SE of 6 separate experiments. \**Myo*-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with control; †*myo*-inositol accumulation significantly increased ( $P < 0.05$ ) compared with TNF- $\alpha$ -treated cells.

incorporation into cellular proteins. For these studies, BAE or murine CME cells were incubated in serum-free medium in the absence or presence of TNF- $\alpha$  (5 ng/ml) for 16 h followed by a 6-h incubation in serum-free medium containing [methyl- $^3$ H]choline chloride or L-[4,5- $^3$ H]leucine. Results from these studies showed that choline accumulation and incorporation into phospholipid and L-leucine accumulation and incorporation into cellular proteins were not altered by TNF- $\alpha$  (data not shown). In other studies, we examined the accumulation of choline and leucine over a 15- to 60-min incubation period after treatment of BAE, bovine PA endothelial, or CME cells with or without TNF- $\alpha$  for 16 h. These studies also indicated that TNF- $\alpha$  did not alter choline or leucine accumulation (data not shown). Therefore, the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation is apparently not due to a general defect in the cellular uptake of phospholipid or protein synthesis substrates. However, it must be noted that these studies did not examine the effect of TNF- $\alpha$  on the initial rate of choline or leucine uptake by the cells.

It is unlikely that the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation is a secondary event due to TNF- $\alpha$ -induced apoptosis (12, 23). In a study using neutral red, no significant difference between the uptake of the dye by normal or TNF- $\alpha$ -treated cells was observed. BAE cells treated with 0.1, 0.5, or 5.0 ng/ml TNF- $\alpha$  for 16 h absorbed  $113 \pm 9$ ,  $84 \pm 9$ , and  $79 \pm 11\%$  ( $P > 0.30$ ) of the dye compared with normal cells. In addition, there was no change in the amount of neutral red absorbed by murine CME cells treated with 5 ng/ml TNF- $\alpha$  compared with control cells (data not shown).

Table 1. Effect of TNF- $\alpha$  on *myo*-inositol accumulation and incorporation into phospholipids and intracellular *myo*-inositol content in bovine aorta endothelial cells

Conditions	Myo-Inositol, nmol/mg protein		
	Accumulation	Incorporation into phosphoinositides	Intracellular content
Control	$3.50 \pm 0.22$	$1.34 \pm 0.14$	$13.6 \pm 3.7$
TNF- $\alpha$	$1.44 \pm 0.06^*$	$0.77 \pm 0.03^*$	$3.7 \pm 1.1^*$

Data are presented as mean  $\pm$  SE of 9 separate determinations. Cells were incubated in serum-free medium for 16 h in absence (control) or presence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (5 ng/ml). Afterwards, accumulation and incorporation of extracellular *myo*-inositol into phosphoinositides were determined by incubating cells for an additional 6 h in fresh serum-free medium containing  $11.4 \mu$ M *myo*-[2- $^3$ H]inositol. Another set of cells was used to determine free intracellular *myo*-inositol content by gas chromatography after a 24-h incubation in serum-free medium in absence or presence of TNF- $\alpha$  (5 ng/ml). \* $P < 0.05$  compared with control.



**Effect of TNF- $\alpha$  and/or hyperosmolarity on myo-inositol accumulation.** Previously, we had shown that exposing cultured endothelial cells to hyperosmotic conditions by the addition of 150 mM raffinose (~490 mosM) to serum-free medium for 6–24 h caused an increase in myo-inositol accumulation that was preceded by an increase in SMIT mRNA levels (36). Because TNF- $\alpha$  and hyperosmolarity apparently have contrasting effects on myo-inositol accumulation in cultured endothelial cells, we examined the effect of TNF- $\alpha$  on the hyperosmolarity-induced increase in myo-inositol accumulation. For these studies, murine CME, BAE, or bovine PA endothelial cells were exposed to serum-free medium containing 5 ng/ml TNF- $\alpha$  (T), 150 mM raffinose (R) or a combination of the two (T + R) for 16 h. The cells receiving TNF- $\alpha$  and raffinose (T + R) were preincubated with TNF- $\alpha$  for 1 h before the addition of raffinose. Afterwards, myo-inositol accumulation was determined by incubating the cells for 1 h in osmotically matched buffer containing 10  $\mu$ M myo-[2- $^3$ H]inositol. Figure 6 shows that hyperosmolarity (R) increased myo-inositol accumulation compared with control in all three types of endothelial cells, whereas TNF- $\alpha$  caused a significant decrease in myo-inositol accumulation. The addition of TNF- $\alpha$  to the hyperosmotic medium reduced the hyperosmolarity-induced increase in myo-inositol accumulation by 30–50%.

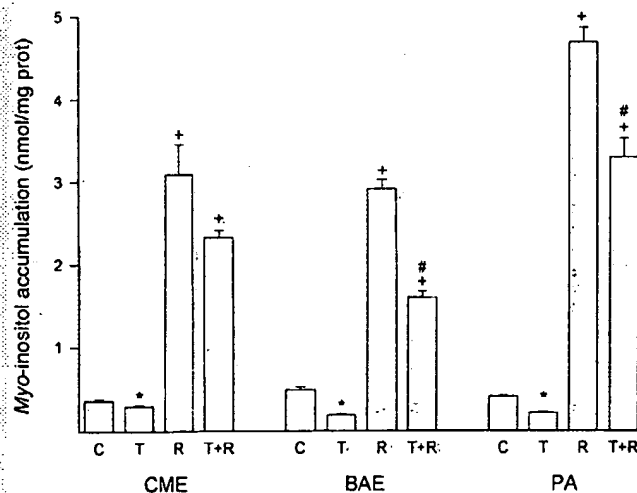


Fig. 6. Effect of TNF- $\alpha$ , hyperosmolarity, or combination of TNF- $\alpha$  and hyperosmolarity on myo-inositol accumulation. Murine CME, BAE, and bovine PA endothelial cells were grown in 6-well plates and then incubated for 16 h in serum-free medium (C) or same medium containing TNF- $\alpha$  (T, 5 ng/ml), 150 mM raffinose (R, ~490 mosM) or combination of TNF- $\alpha$  and 150 mM raffinose (T + R). Osmolarity of isotonic medium was ~310 mosM. Cells that received combination of TNF- $\alpha$  and 150 mM raffinose were preincubated for 1 h with TNF- $\alpha$  before addition of raffinose. Myo-inositol accumulation was then determined by incubating cells for 1 h in osmotically matched HEPES buffer containing 10  $\mu$ M myo-inositol. Myo-inositol accumulation is expressed as nmol/mg protein (prot). Each value is mean  $\pm$  SE of 9 separate experiments. \*Myo-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with C; \*myo-inositol accumulation significantly increased ( $P < 0.05$ ) compared with C; \*myo-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with hyperosmotic treated cells (R).

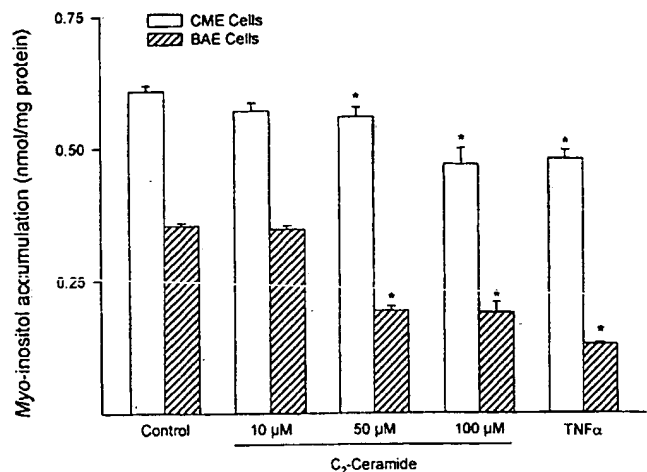


Fig. 7. Effect of C<sub>2</sub>-ceramide on myo-inositol accumulation. Murine CME or BAE cells were grown in 6-well plates and then incubated for 16 h in serum-free medium (control) or same medium containing 10–100  $\mu$ M C<sub>2</sub>-ceramide or 5 ng/ml TNF- $\alpha$ . Myo-inositol accumulation was then determined by incubating cells for 1 h in serum-free medium containing myo-[2- $^3$ H]inositol. Myo-inositol accumulation is expressed as nmol/mg protein. Each value is mean  $\pm$  SE of 9 separate experiments. \*Myo-inositol accumulation significantly decreased ( $P < 0.05$ ) compared with control.

**Effect of inhibitors, LPS, C<sub>2</sub>-ceramide, and sphingosine on myo inositol accumulation.** Incubation of BAE cells with TNF- $\alpha$  (5 ng/ml) for 10 min caused a fourfold increase in ceramide levels [ $75.8 \pm 6.7$  (control cells) to  $318.9 \pm 28.7$  pmol/nmol lipid phosphorus, (TNF- $\alpha$ -treated cells)  $P < 0.05$ ,  $n = 6$ ]. Ceramide levels were also significantly increased by about twofold in CME cells (data not shown). To determine whether the generation of ceramide was responsible for the TNF- $\alpha$ -induced decrease in myo-inositol accumulation, we examined the effect of C<sub>2</sub>-ceramide on myo-inositol accumulation by murine CME and BAE cells and compared it to the effect of TNF- $\alpha$ . For these studies, the cells were incubated for 16 h in serum-free medium containing 10–100  $\mu$ M C<sub>2</sub>-ceramide or 5 ng/ml TNF- $\alpha$ . Afterwards, myo-inositol accumulation was determined by incubating the cells for 1 h in serum-free medium containing myo-[2- $^3$ H]inositol. Data in Fig. 7 demonstrate that C<sub>2</sub>-ceramide, in a concentration-dependent fashion, caused a significant decrease in myo-inositol accumulation by murine CME and BAE cells. These results suggest that ceramide production may be mediating the effect of TNF- $\alpha$  on myo-inositol accumulation. In contrast to the effect of C<sub>2</sub>-ceramide on myo-inositol accumulation, incubating BAE, bovine PA endothelial, or CME cells for 16 h in serum-free medium containing sphingosine (50  $\mu$ M) inhibited myo-inositol accumulation by <10% in bovine PA endothelial and CME cells and by <15% in BAE cells (data not shown). None of these changes achieved statistical significance.

As mentioned above, TNF- $\alpha$  activates a variety of signal transduction pathways, including PKC, PLA<sub>2</sub>, and protein phosphorylation of serine-threonine and tyrosine residues (9, 11, 27, 28, 35). To determine the signal transduction pathway(s) responsible for the TNF-

**Table 2. *Myo*-inositol accumulation by bovine aortic endothelial cells: effect of lipopolysaccharide and inhibitors of kinases, phosphatases, NF- $\kappa$ B activation, phospholipase A<sub>2</sub>, and ceramidase on TNF- $\alpha$  activity**

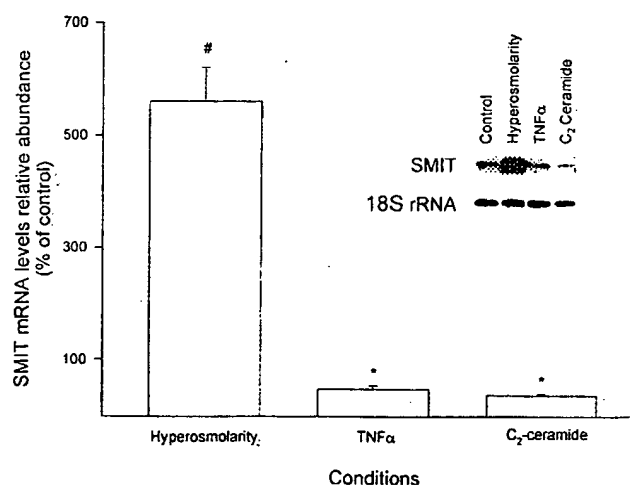
Conditions	n	Myo-Inositol Accumulation Before and After TNF- $\alpha$ (5 ng/ml)	
		-	+
Control	14	100-	52 $\pm$ 2*
Pyrrolidinedithiocarbamate (100 $\mu$ M)	9	170 $\pm$ 21	133 $\pm$ 18†
TLCK (50 $\mu$ M)	9	140 $\pm$ 14	107 $\pm$ 14†
PD-98059 (500 nM)	6	93 $\pm$ 9	50 $\pm$ 4*†
SB-203580 (25 $\mu$ M)	6	111 $\pm$ 6	63 $\pm$ 2*†
Rapamycin (10 nM)	6	72 $\pm$ 3*	49 $\pm$ 4*†
Wortmannin (100 nM)	6	88 $\pm$ 2	45 $\pm$ 4*†
Genistein (50 $\mu$ M)	12	115 $\pm$ 18	93 $\pm$ 4†
Orthovanadate (25 $\mu$ M)	9	125 $\pm$ 2	64 $\pm$ 8*†
Calphostin C (1 $\mu$ M)	9	113 $\pm$ 11	84 $\pm$ 2†
Staurosporin (100 nM)	9	82 $\pm$ 2	57 $\pm$ 5*†
Lipopolysaccharide (1 $\mu$ g/ml)	6	66 $\pm$ 1*	57 $\pm$ 2*
N-Oleylethanolamine (0.5 mM)	6	98 $\pm$ 3	55 $\pm$ 3*†
4-Bromophenacyl bromide (10 $\mu$ M)	6	103 $\pm$ 4	48 $\pm$ 2*†

Data are presented as means  $\pm$  SE of %control; n = no. of determinations. Cells were preincubated for 1 h in serum-free medium in absence or presence of each of the compounds before the addition of TNF- $\alpha$  (-). After addition of TNF- $\alpha$  (+), incubations were continued for an additional 15 h. Afterwards, *myo*-inositol accumulation was determined by incubating cells for 1 h in serum-free medium containing *myo*-[2-<sup>3</sup>H]inositol. TLCK, 7-amino-2-chloro-3-toysylamido-2-heptanone. \**P* < 0.05 compared with control; †*P* < 0.05 compared with TNF- $\alpha$ -treated control cells; ‡*P* < 0.05 compared with treatment alone.

$\alpha$ -induced inhibition of *myo*-inositol accumulation, we examined the effect of various inhibitors of these signal transduction pathways and inhibitors of phosphorylation, protease degradation, and phosphatases on the TNF- $\alpha$ -induced inhibition of *myo*-inositol accumulation in BAE cells (Table 2). In many cells, the effect of TNF- $\alpha$  is mediated through the activation of the transcription factor NF- $\kappa$ B, which is activated by the degradation of I- $\kappa$ B protein (4). Therefore, we also examined the effect of inhibitors of NF- $\kappa$ B activation on the TNF- $\alpha$ -induced inhibition of *myo*-inositol accumulation in BAE cells. Inhibitors of ERK1 (PD-98059), p38 kinase (SB-203580), p70 S6 kinase (rapamycin), or phosphatidylinositol 3-kinase (wortmannin) did not affect the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. Pyrrolidinedithiocarbamate, an inhibitor of NF- $\kappa$ B activation, blocked the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. TLCK, a protease inhibitor used to block I- $\kappa$ B- $\alpha$  degradation, prevented the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. Genistein, a protein tyrosine kinase inhibitor used to block the phosphorylation of I- $\kappa$ B- $\alpha$ , partially blocked the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. Orthovanadate, a protein tyrosine phosphatase inhibitor, did not affect the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. Activation of PKC- $\zeta$  has been reported to regulate the binding of NF- $\kappa$ B to DNA in nuclear extracts, possibly through the phosphorylation and subsequent degradation of I- $\kappa$ B- $\alpha$  (26). Calphostin C has been shown to inhibit PKC- $\zeta$ , whereas staurospo-

rine, a nonspecific inhibitor of PKC, does not inhibit PKC- $\zeta$  (26). Consistent with these observations, calphostin C, but not staurosporine, partially inhibited the effect of TNF- $\alpha$  on *myo*-inositol accumulation. N-oleylethanolamine and 4-bromophenacyl bromide, inhibitors of ceramidase and PLA<sub>2</sub> activity, respectively, had no effect on the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. LPS mimicked the effect of TNF- $\alpha$  on *myo*-inositol accumulation, and their combined effects were not additive.

**SMIT mRNA levels.** Given our previous results, which stated that the effect of hyperosmolarity on *myo*-inositol accumulation occurred at the level of SMIT gene expression (36), we next examined the effect of TNF- $\alpha$  on SMIT mRNA levels. Data in Fig. 8 demonstrate the effect of hyperosmolarity, TNF- $\alpha$ , and C<sub>2</sub>-ceramide on SMIT mRNA levels in murine CME cells. As previously reported, hyperosmolarity stimulated a large increase in SMIT mRNA levels compared with the level in control cells incubated in isotonic medium (36). In contrast, treatment with TNF- $\alpha$  and C<sub>2</sub>-ceramide significantly decreased SMIT mRNA levels by 50–70%. The insert shows a representative autoradiograph of SMIT mRNA and 18S rRNA. Examination of the time course of the effect of TNF- $\alpha$  and C<sub>2</sub>-ceramide on SMIT mRNA levels in murine CME cells demonstrated that a maximum decrease in SMIT mRNA levels occurred after a 6-h incubation with TNF- $\alpha$  or C<sub>2</sub>-ceramide (data not shown). Analysis of SMIT mRNA levels in BAE cells by Northern blot



**Fig. 8. Effect of hyperosmolarity, TNF- $\alpha$ , and C<sub>2</sub>-ceramide on Na<sup>+</sup>-*myo*-inositol cotransporter (SMIT) mRNA levels in murine CME cells.** Cells were grown in 75-cm<sup>2</sup> flasks to confluency and then incubated for 6 h in either serum-free medium or medium containing TNF- $\alpha$  (5 ng/ml), C<sub>2</sub>-ceramide (50  $\mu$ M), or 150 mM raffinose (~490 mosM). Afterwards, RNA was isolated and SMIT mRNA levels were determined as described in MATERIALS AND METHODS. SMIT mRNA levels were standardized using 18S rRNA levels. Data are presented as a percentage of control, with level of SMIT mRNA in control cells assigned a value of 1. Each value is mean  $\pm$  SE of a minimum of 5 separate determinations. *Inset* shows a representative autoradiograph of levels of SMIT mRNA and 18S rRNA. \*SMIT mRNA levels significantly decreased (*P* < 0.05) compared with control, †SMIT mRNA levels significantly increased (*P* < 0.05) compared with control.

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analysis demonstrated that SMIT mRNA levels in cells exposed to TNF- $\alpha$  (5 ng/ml) for 16 h were reduced to 20% of control levels, whereas SMIT mRNA levels in cells exposed to hyperosmotic medium for 16 h were increased over 10-fold, after correction for  $\beta$ -actin mRNA levels (Fig. 9).

**Electrophoretic mobility shift assay.** The above data suggest that the effect of TNF- $\alpha$  on *myo*-inositol accumulation is associated with the activation of NF- $\kappa$ B. To determine whether TNF- $\alpha$  activated NF- $\kappa$ B in BAE and bovine PA endothelial cells, the ability of TNF- $\alpha$  to stimulate NF- $\kappa$ B binding to its consensus DNA binding site was examined. Data in Fig. 10 demonstrate that a 15- or 30-min incubation with TNF- $\alpha$  (5 ng/ml) increased NF- $\kappa$ B in nuclear extracts from BAE or bovine PA endothelial cells. In contrast, an oligonucleotide probe containing a mutated NF- $\kappa$ B consensus sequence did not produce a gel-shifted band when nuclear extracts from TNF- $\alpha$ -treated cells were used (data not shown). DNA protein binding for an unrelated probe containing the consensus sequence for the E-box of the adenovirus major late transcription factor promoter was not affected by TNF- $\alpha$  treatment (Fig. 10). In contrast, treating BAE or bovine PA endothelial cells for 15 or 30 min with hyperosmotic medium had no effect on NF- $\kappa$ B activation (data not shown). Data in Fig. 11 show that a 10- or 50-fold excess of unlabeled NF- $\kappa$ B oligonucleotide competed for binding of the NF- $\kappa$ B-labeled probe in nuclear extracts prepared from BAE and bovine PA endothelial cells treated with TNF- $\alpha$ . The mutant NF- $\kappa$ B oligonucleotide exhibited weak competition for the NF- $\kappa$ B gel shift, and a nonspecific unlabeled oligonucleotide containing the E-box of the adenovirus major late transcription factor promoter had no effect on NF- $\kappa$ B oligonucleotide binding. As shown in Table 2, we found that calphostin C, genistein, pyrrolidinedithiocarbamate, and TLCK, each to varying degrees, were able to prevent the TNF- $\alpha$ -induced inhibition of *myo*-inositol accumulation by BAE cells. To determine whether the effects of these compounds

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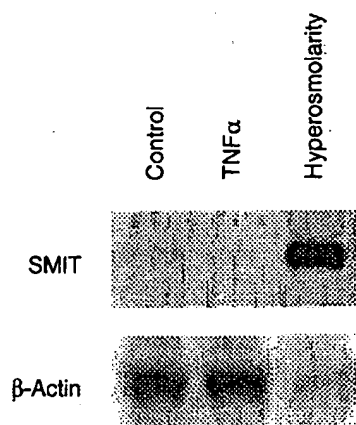


Fig. 9. Northern blot analysis of BAE cell SMIT mRNA levels: effect of TNF- $\alpha$  and hyperosmolarity. Cells were grown in 75-cm<sup>2</sup> flasks to confluency and then incubated for 16 h in serum-free medium or same medium containing TNF- $\alpha$  (5 ng/ml) or 150 mM raffinose (~490 mosM). RNA was isolated, and SMIT and  $\beta$ -actin mRNA levels were determined as described in MATERIALS AND METHODS.

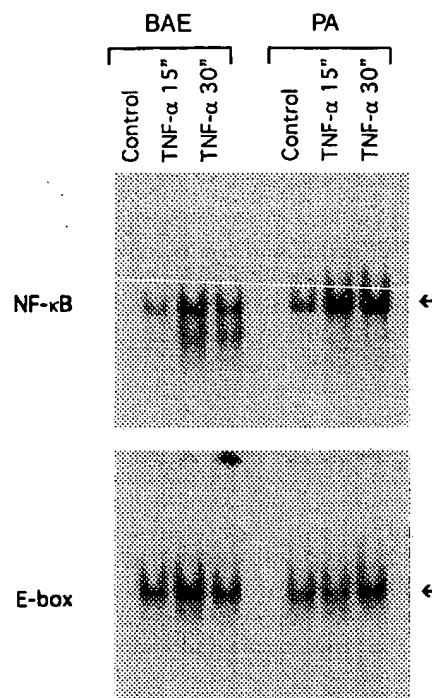


Fig. 10. Electrophoretic mobility shift assay of nuclear factor (NF)- $\kappa$ B, using nuclear extracts from BAE and bovine PA endothelial cells. Cells were grown in 150-cm<sup>2</sup> flasks to confluency and then incubated for 15 (15") or 30 (30") min in medium with or without 5 ng/ml TNF- $\alpha$ . Cells were then harvested, nuclear extracts were prepared, and gel mobility shift assays were performed as described in MATERIALS AND METHODS. For these studies, radiolabeled oligonucleotide probes containing the consensus sequence for NF- $\kappa$ B (top) or E-box of the adenovirus major late transcription factor promoter (bottom) were used.

may be related to blocking the TNF- $\alpha$ -induced activation of NF- $\kappa$ B, we conducted an electrophoretic mobility-shift assay using nuclear extracts prepared from untreated and TNF- $\alpha$ -treated (5 ng/ml, 30 min) cells as well as from cells pretreated for 1 h with each of these inhibitors followed by a 30-min incubation with TNF- $\alpha$ . Data in Fig. 12 (representative of 3 separate experiments) show that TNF- $\alpha$  activation of NF- $\kappa$ B in BAE cells is partially reduced by calphostin C and pyrrolidinedithiocarbamate and totally blocked by TLCK. Genistein appeared not to decrease TNF- $\alpha$  activation of NF- $\kappa$ B. NF- $\kappa$ B is a member of the Rel family of transcriptional regulatory proteins which includes p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), Rel A (p65), cRel, and Rel B. The existence of more than one of these members in a cell may result in the appearance of more than one NF- $\kappa$ B binding complex. Data in Fig. 10 show that nuclear extracts from TNF- $\alpha$ -treated BAE cells form two complexes, a faster-migrating lower complex and a predominant upper complex. This may be due to the presence of more than one isoform of NF- $\kappa$ B in BAE cells. However, this has not been a consistent finding, as shown in Fig. 13. Data in Fig. 13 show that in nuclear extracts prepared from BAE and bovine PA endothelial cells treated with TNF- $\alpha$  for 30 min, the p65 antibody caused a gel retardation (supershift) in

the binding complex. In contrast, p50 antibody caused only a minimal gel retardation. As a control, data in Fig. 13 show that an antibody to CREB had no effect. These results suggest that p65 is the major NF- $\kappa$ B form activated by TNF- $\alpha$  in these cells.

## DISCUSSION

Most mammalian cells acquire *myo*-inositol through an active, Na<sup>+</sup>-dependent cotransporter (SMIT), and the internal concentration of *myo*-inositol is generally 5- to 500-fold higher than the level of *myo*-inositol in plasma or extracellular fluid (2). *Myo*-inositol is an important component of membranes and, in the form of phosphoinositides, is an integral part of several signal transduction pathways (1, 20, 21). In addition, in renal and other cells, *myo*-inositol is one of several organic osmolytes that are important for maintaining an osmotic balance during periods of osmotic stress (3, 36).

Increased osmotic stress activates different members of the MAP kinase family of signal transduction proteins, including ERK, JNK, and p38 kinase (14, 15). Cytokines, such as TNF- $\alpha$ , have been shown to regulate the activity of these same signal transduction proteins (14, 15). Therefore, we designed studies to determine the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation, anticipating that the effect of TNF- $\alpha$  on *myo*-inositol accumulation would be similar to the effect of hyperosmolarity. However, in contrast to the effect of hyperosmolarity, TNF- $\alpha$  significantly de-

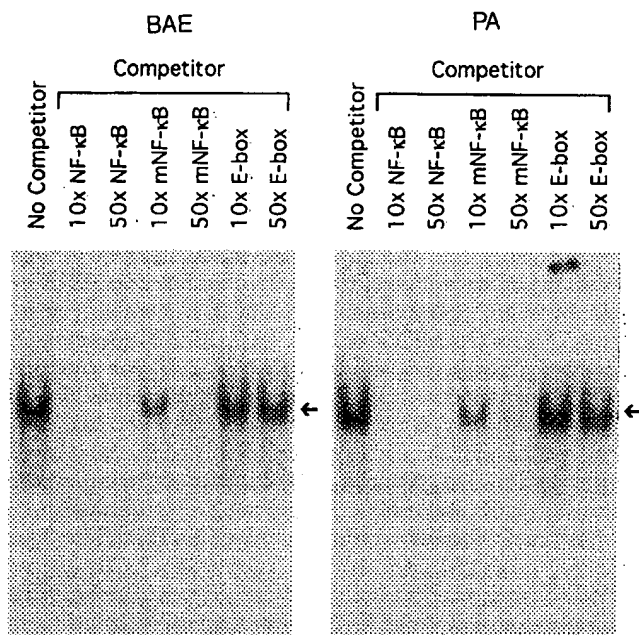


Fig. 11. Competition electrophoretic mobility shift assay of NF- $\kappa$ B. Nuclear extracts prepared from BAE and bovine PA endothelial cells treated for 30 min with 5 ng/ml TNF- $\alpha$  were used to determine binding competition by a 10- (10 $\times$ ) or 50-fold (50 $\times$ ) excess of unlabeled oligonucleotide for NF- $\kappa$ B, mutated (m) NF- $\kappa$ B, or E-box of the adenovirus major late transcription factor promoter, using radiolabeled oligonucleotide probe containing the consensus sequence for NF- $\kappa$ B. Gel mobility shift assay was performed as described in MATERIALS AND METHODS.

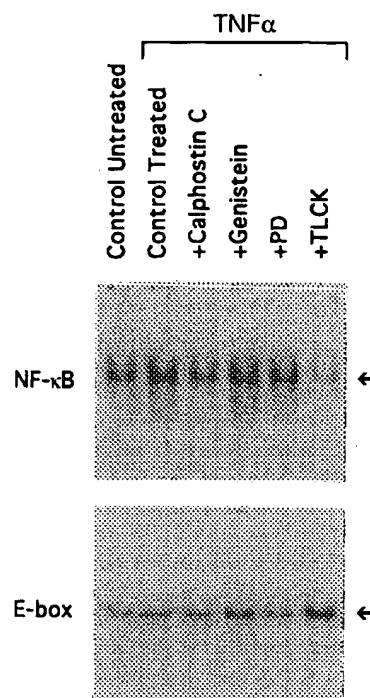


Fig. 12. Effect of calphostin C, genistein, pyrrolidinedithiocarbamate (PD), and 7-amino-1-chloro-3-tosylamido-2-heptanone (TLCK) on TNF- $\alpha$ -induced activation of NF- $\kappa$ B in BAE cells. Cells were grown to confluency in 150-cm<sup>2</sup> flasks and then incubated for 30 min with TNF- $\alpha$  (5 ng/ml) in absence or presence of inhibitors. Cells receiving inhibitors were preincubated for 1 h with each inhibitor before addition of TNF- $\alpha$ . Afterwards, cells were harvested, nuclear extracts were prepared, and gel mobility shift assays were performed as described in MATERIALS AND METHODS. For these studies, radiolabeled oligonucleotide probes containing the consensus sequence for NF- $\kappa$ B (top) or E-box of the adenovirus major late transcription factor promoter (bottom) were used.

creased *myo*-inositol accumulation and SMIT mRNA levels in cultured large-vessel endothelial cells and CME cells. The effect of TNF- $\alpha$  on *myo*-inositol accumulation was mimicked by LPS, IL-1 $\beta$ , and TGF- $\beta$  in large-vessel endothelial cells. In addition, the effect of TNF- $\alpha$  on *myo*-inositol accumulation was highly selective, in that TNF- $\alpha$  did not decrease the accumulation of *myo*-inositol by a wide variety of other mammalian-cultured cells, including microvessel endothelial cells derived from periaortic adipose tissue and coronary artery endothelial cells. The reason for these differences is not known. However, it seems unlikely that the lack of an effect by TNF- $\alpha$  on *myo*-inositol accumulation by the unaffected cells was due to an absence of TNF- $\alpha$  receptors, because TNF- $\alpha$  interacts with a wide variety of cell types and most cells express specific TNF- $\alpha$  receptors on their cell surface (12).

Stimulation of sphingomyelinase by TNF- $\alpha$  causes the release of two second messengers, ceramide and sphingosine (19, 27, 28, 35). Our studies showed that the inhibition of sphingosine production by *N*-oleoyl-ethanolamine did not influence the effect of TNF- $\alpha$  on *myo*-inositol accumulation (26). In addition, incubating endothelial cells with sphingosine had no significant effect on *myo*-inositol accumulation. In contrast, the

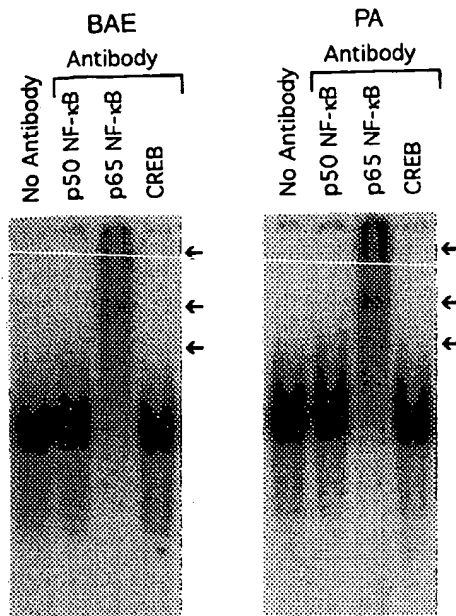


Fig. 13. Electrophoretic mobility supershift assay of NF- $\kappa$ B. BAE and bovine PA endothelial cells were grown to confluency in 150-cm<sup>2</sup> flasks and then incubated for 30 min with TNF- $\alpha$  (5 ng/ml). Afterwards, cells were harvested, nuclear extracts were prepared, and gel mobility supershift assays were performed as described in MATERIALS AND METHODS. For these studies, nuclear extracts were preincubated with or without 1  $\mu$ g of antibodies anti-p50, anti-p65, or anti-CREB before addition of radiolabeled oligonucleotide probe containing the consensus sequence for NF- $\kappa$ B.

addition of C<sub>2</sub>-ceramide to the medium mimicked the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation. These results suggest that the production of ceramide is responsible for the TNF- $\alpha$ -induced decrease in SMIT mRNA levels and *myo*-inositol accumulation.

The activation of ERK1, p38 kinase, S6 kinase, phosphatidylinositol 3-kinase, or PLA<sub>2</sub> by TNF- $\alpha$  are not necessary for the inhibition of *myo*-inositol accumulation by TNF- $\alpha$ . Studies by Kwon et al. (13) have indicated that activation of the MAP kinase cascade does not contribute to the hyperosmotic induction of *myo*-inositol or betaine uptake by Madin-Darby canine kidney cells. Taken together, these studies suggest that the activation of the MAP kinase cascade may not contribute to the regulation of *myo*-inositol accumulation by osmotic stress or cytokines. However, it remains to be determined whether the activation of JNK may have a role in mediating *myo*-inositol accumulation by TNF- $\alpha$ . Lee et al. (16) have shown that in HeLa cells TNF- $\alpha$  activates MAP kinase/ERK kinase 1, a kinase in the pathway of JNK activation. JNK, in turn, have also been shown to phosphorylate I- $\kappa$ B- $\alpha$ , resulting in activation of NF- $\kappa$ B.

Our data show that TNF- $\alpha$  activates NF- $\kappa$ B in BAE and bovine PA endothelial cells and that the activation of NF- $\kappa$ B is associated with the effect of TNF- $\alpha$  on SMIT mRNA levels and *myo*-inositol accumulation. Staurosporine, a nonspecific inhibitor of PKC, did not affect the decrease in *myo*-inositol accumulation by TNF- $\alpha$  (26).

In contrast, the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation was partially prevented by calphostin C, which inhibits PKC- $\zeta$ , an isoform of PKC that is insensitive to staurosporine and has been shown to stimulate binding of NF- $\kappa$ B to DNA (26). Moreover, pyrrolidinedithiocarbamate, an inhibitor of NF- $\kappa$ B activation, and genistein and TLCK, a protein tyrosine kinase and protease inhibitor, respectively, also significantly reduced the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation. These latter two agents could inhibit the proteolytic degradation of I- $\kappa$ B and thereby inhibit the activation of NF- $\kappa$ B. Consistent with their effects on *myo*-inositol accumulation, TLCK, calphostin C, and pyrrolidinedithiocarbamate prevented the TNF- $\alpha$ -induced activation of NF- $\kappa$ B to different degrees in BAE cells. In contrast, genistein, which partially prevented the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation, did not prevent the TNF- $\alpha$ -mediated activation of NF- $\kappa$ B. The reason for this inconsistency is unknown and requires further investigation. Nonetheless, these data suggest that TNF- $\alpha$  activates NF- $\kappa$ B by a mechanism that could be dependent on the activation of PKC- $\zeta$  and that NF- $\kappa$ B, in turn, may directly or indirectly decrease the expression of the SMIT gene and reduce SMIT protein levels. TNF- $\alpha$  has been shown to repress transcription of several genes, including the vascular endothelial growth factor receptor gene in endothelial cells (22) and the CCAAT-enhancer binding protein and glucose transporter isoform 4 genes in 3T3-L1 adipocytes (30). However, it has not been shown that the activation of NF- $\kappa$ B by TNF- $\alpha$  is directly responsible for downregulating the expression of these genes or the SMIT gene. NF- $\kappa$ B is primarily an activator of gene transcription. It is possible that TNF- $\alpha$ -induced activation of NF- $\kappa$ B is an independent event and not related to the TNF- $\alpha$ -mediated regulation of SMIT mRNA levels or *myo*-inositol accumulation. Second, it is possible that NF- $\kappa$ B activation is increasing the transcription of a protein(s) that regulates SMIT mRNA stability and/or protein turnover and thus is a secondary event contributing to the posttranscriptional regulation of the SMIT. To unequivocally answer this question, nuclear run-on assays are currently being conducted as well as studies of the 5'-flanking region of the SMIT gene, which has not yet been completely sequenced (25). Therefore, at this time we can only state that the downregulation of SMIT mRNA levels and *myo*-inositol accumulation by TNF- $\alpha$  parallels the activation of NF- $\kappa$ B in endothelial cells.

In summary, our studies show that TNF- $\alpha$  regulates SMIT mRNA levels and *myo*-inositol accumulation in cultured large-vessel endothelial cells and CME cells. TNF- $\alpha$ -induced ceramide production appears to be a proximal event in this effect, whereas the studies demonstrating that inhibitors of NF- $\kappa$ B activation partially prevent the TNF- $\alpha$ -induced decrease in *myo*-inositol accumulation suggest that a distal event in the effect of TNF- $\alpha$  may include NF- $\kappa$ B activation. Elucidation of the signaling pathways linking NF- $\kappa$ B activation with ceramide production and regulation of SMIT mRNA levels and *myo*-inositol accumulation will re-



quire further investigation. The contrasting effects of TNF- $\alpha$  and hyperosmolarity on the regulation of SMIT mRNA levels and *myo*-inositol accumulation suggest separate regulatory pathways. One difference is that hyperosmolarity, unlike TNF- $\alpha$ , does not activate NF- $\kappa$ B. To date, the 5'-flanking region of the SMIT gene has not been cloned, and it is unknown whether the putative "osmotic response element" (ORE) of the SMIT gene is similar to the ORE of the aldose reductase gene or the tonicity-sensitive element of the betaine transporter gene (6, 25, 31). Additional studies, such as cloning and characterization of the 5'-flanking region of the SMIT gene, will be necessary to advance our understanding of the regulation of *myo*-inositol metabolism. Interestingly, TNF- $\alpha$  has a variety of effects in endothelial cells, including the regulation of the expression of cell adhesion molecules, proteoglycan synthesis, cell permeability, and proliferation (4, 10). Whether changes in *myo*-inositol metabolism are associated with these TNF- $\alpha$ -mediated events in endothelial cells is currently under investigation. As mentioned previously, *myo*-inositol metabolism is associated with membrane synthesis and is required for signal transduction pathways, so changes in *myo*-inositol metabolism could have a wide range of effects on the endothelium.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-45453 and DK-25295 and by a Merit Review Grant from the Department of Veterans Affairs.

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Received 2 January 1997; accepted in final form 3 September 1997.

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